CHAPTER 2

MATERIALS AND METHODS

2.1 Materials and equipments

2.1.1 Chemicals

- Absolute ethanol (Liquor Distillery Organization, Excise Department, Ministry of Finance, Chachoengsao, Thailand)
- Acetic acid (RCI Labscan Ltd., Bangkok, Thailand)
- Ammonium molybdate (Fisher Scientific UK Ltd., Loughborough, UK)
- Anhydrous sodium sulfate (Ajax Finechem, New South Wales, Australia)
- Bovine serum albumin (Amresco, Ohio, USA)
- Cholesterol (Fluka AG, Buchs, Switzerland)
- Coomassie brilliant blue G-250 (Bio-Rad Laboratories, Inc., Herculues, CA, USA)
- Dicetyl phosphate (Sigma-Aldrich, St. Louis, MO, USA)
- Dichloromethane (RCI Labscan Ltd., Bangkok, Thailand)
- Diethyl ether (RCI Labscan Ltd., Bangkok, Thailand)
- Dimethyl dioctadecyl ammonium bromide (Sigma-Aldrich, St. Louis, MO, USA)
- Disodium hydrogen phosphate (Merck, Darmstadt, Germany)
- *L*-Dopa (Sigma-Aldrich, St. Louis, MO, USA)
- Dulbecco’s modified Eagle medium (GIBCO, Maryland, USA)

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- Ethyl acetate (RCI Labscan Ltd., Bangkok, Thailand)
- Ethylenediamine tetraacetic acid (Merck, Darmstadt, Germany)
- Fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria)
- Hexane (RCI Labscan Ltd., Bangkok, Thailand)
- Lauric acid (Acros Organics, NJ, USA)
- Lysis reagent (Fermentas, EU)
- Methanol (RCI Labscan Ltd., Bangkok, Thailand)
- Mushroom tyrosinase (Sigma-Aldrich, St. Louis, MO, USA)
- Myristic acid (Acros Organics, NJ, USA)
- Palmitic acid (Acros Organics, NJ, USA)
- Palmitic acid methyl ester (Sigma-Aldrich, St. Louis, MO, USA)
- Penicillin/streptomycin solution (GIBCO, Maryland, USA)
- Pentobarbitone sodium (Sanofi, Brussels, Belgium)
- Phosphomolybdic acid (Carlo Erba Reagents, Rodano, Italy)
- Propylene glycol (Jindecheng Trading Co., Ltd., China)
- Propylene glycol/diazolidinyl urea/iodopropynyl butylcarbamate (Liquid germall plus®) (International Specialty Products, Wayne, NJ, USA)
- Protease inhibitors (Roche, Germany)
- Polyoxyethylene-4-sorbitan monostearate (Tween61) (Sigma Chemical Co., St. Louise, MO, USA)
- Polyoxyethylene-2-stearyl ether (Brij72) (Fluka AG, Buchs, Switzerland)
- Sephadex G-50 (Sigma-Aldrich, St. Louis, MO, USA)
- Silica gel G60 (Merck, Darmstadt, Germany)
- Sodium bicarbonate (Ajax Finechem, New South Wales, Australia)

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- Sodium dihydrogen phosphate (Merck, Darmstadt, Germany)
- Sodium hydroxide (Merck, Darmstadt, Germany)
- Sodium periodate (Rankem, Delhi, India)
- Sorbitan monostearate (Span60) (Sigma Chemical Co., St. Louise, MO, USA)
- Stearic acid (Acros Organics, NJ, USA)
- Sulforhodamine B monosodium salt (Sigma-Aldrich, St. Louis, MO, USA)
- Sulfuric acid (RCI Labscan Ltd., Bangkok, Thailand)
- Theophylline (Sigma-Aldrich, St. Louis, MO, USA)
- Trichloroacetic acid (Merck, Darmstadt, Germany)
- Tris (hydroxymethyl) methylamine (Merck, Darmstadt, Germany)
- Trypsin (Gibco, Grand Island, NY, USA)
- Vitamin E (Sigma-Aldrich, St. Louis, MO, USA)

2.1.2 Cell lines

- B16F10 melanoma cells (ATCC CRL-6475, Manassas, VA, USA)
- Human skin fibroblasts (Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand)

2.1.3 Animals

- C57BL/6 male mice (National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand)
- New Zealand white male rabbits (Faculty of Agriculture, Chiang Mai

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University, Chiang Mai, Thailand)

- Male newborn landrace pigs (Yupa Farm, Chiang Mai, Thailand)

2.1.4 Equipments

- Carbon dioxide incubator (Contherm Scientific Ltd., model 4400 C, New Zealand)
- Centrifuge (Hettich Zentrifugen, model Universal 32R, Germany)
- Fractional collector (Foxy JR, Isco Inc., Lincoln, USA)
- Fourier transform infrared spectrometer (Thermo Fisher Scientific, model Nicolet 5700, Waltham, USA)
- Gas chromatography-mass spectrometer (Agilent Technologies, CA, USA)
- High performance liquid chromatography (Thermo Finnigan, model AS 1000, San Jose, CA, USA)
- Lyophilizer (Christ FOC-1, model K-40 equipment, Balzers-Pfeiffer GmbH, Asslar, Germany)
- Laminar air flow cabinet (Equipments Scientifiques & Industries S.A., model Cytair 125, France)
- Mexameter (Courage & Khazaka, model MX 800, Cologne, Germany)
- Microplate reader (Bio-rad, model 680, Japan)
- Modified vertical Franz diffusion apparatus (Crown Bio Scientific Inc., Sommerville, NJ, USA)
- Optical microscope (Olympus Optical Co., Ltd., Tokyo, Japan)
- Particle size analyzer (Malvern Instruments Ltd., model Zetasizer ZS, Malvern, UK)

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- pH meter (Laboratory Benchtop 86502, AZ Instrument Corp., Taiwan)
- Probe sonicator (Vibra Cell^{TM}, Sonics & Materials Inc., Newtown, CT, USA)
- Reverse-phase column (Phenomenex, model Luna C18, USA)
- Rotary evaporator (Buchi, model R-200, Switzerland)
- Rotary viscometer (Myr VR 3000, model V2, Viscotech, Hispania, SL, Spain)
- Stuart Scientific SMP-1 apparatus (Stuart Scientific, UK)
- Transmission electron microscope (JEOL Ltd., model JSM-6335F, Tokyo, Japan)
- UV-vis spectrophotometer (Shimadzu Corporation, model UV-2450, Kyoto, Japan)

2.2 Methods

2.2.1 Preparation of saturated fatty acid methyl esters

2.2.1.1 Synthesis of saturated fatty acid methyl esters

Saturated fatty acid methyl esters were synthesized using the Fischer esterification reaction under the nitrogen atmosphere[56]. Briefly, 10 mM of saturated fatty acids, including lauric acid (LA), myristic acid (MA), palmitic acid (PA) and stearic acid (SA), were dissolved in 10 ml of dichloromethane, mixed with an excess methanol and added with 3 drops of sulfuric acid as an acid catalyst. The mixture was refluxed at 65±2°C for 4 h. The reaction mixture was cooled to room temperature (30±2°C). Twenty milliliters of distilled water was added and then transferred to a separating funnel. The funnel was inverted 30 times and the organic layer was

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collected. The aqueous layer was extracted with 25 ml of dichloromethane for three times. The organic layer was collected, washed with 25 ml of saturated sodium bicarbonate solution and removed the remaining water by the addition of anhydrous sodium sulfate (1 g). The crude methyl ester was filtered and concentrated using a rotary evaporator at 45±2°C.

2.2.1.2 Purification of the crude saturated fatty acid methyl esters

The crude esters, including lauric acid methyl ester (LM), myristic acid methyl ester (MM), palmitic acid methyl ester (PM) and stearic acid methyl ester (SM), were purified by column chromatography using silica gel G60 as the stationary phase. The column was eluted with hexane and ethyl acetate (30:1, v/v) and the fractions were collected at the flow rate of 2 ml/min. Each fraction was detected for the purified ester by thin layer chromatography (TLC) using hexane/diethyl ether/acetic acid (8:2:0.15, v/v) as a mobile phase and sprayed with ethanolic phosphomolybdic acid reagent (10%, w/v). Fractions containing the methyl ester were pooled and the solvents were evaporated under vacuum using a rotary evaporator at 45±2°C. The purified methyl ester was lyophilized to remove the residual water.

2.2.1.3 Identification of the purified saturated fatty acid methyl esters

The purified saturated fatty acid methyl esters were then identified for their chemical structures by Fourier transform infrared (FTIR) spectroscopy and gas chromatography-mass spectroscopy (GC/MS). The FTIR spectra were obtained from sample prepared in the potassium bromide (KBr) disk. The chemical structures of the purified methyl esters were also analyzed by GC/MS on the Hewlett Packard model HP6890 connected to an HP model 5973 mass-selective detector. The HP-5MS (5%

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phenyl-polymethylsiloxane) capillary column (30 m × 0.25 mm i.d.) with the film thickness of 0.25 μm was used. The temperature of the column was run at 80-250°C with the increasing rate of 3°C/min and end at 250°C for 5 min. The injector and detector temperatures were at 220 and 280°C, respectively. Helium was used as a carrier gas at the flow rate of 1 ml/min. The column head pressure was 9.29 psi and the injection volume was 2 μl. The retention time and mass spectra of the purified compounds were identified using the Wiley 275 and NIST 98 databases.

2.2.1.4 Physical characteristics and stability of the purified saturated fatty acid methyl esters

Physical characteristics of methyl esters were investigated for the melting (m.p.) and boiling (b.p.) points by using the Stuart Scientific SMP-1 apparatus and the test-tube method, respectively. Also, the physical and chemical stability of the four methyl esters were evaluated. Briefly, an aliquot of the methyl esters (1 ml of the dry methyl ester) was kept in the vials and stored at 4±2, room temperature (30±2) and 45±2°C for 3 months. At 0, 1, 2 and 3 months, the physical appearances (color and odor) of the samples were observed. The samples were withdrawn for the analysis of methyl ester contents by HPLC using a reverse phase C18 column[197]. The HPLC was operated in the linear gradient with mobile phase A (water) and B (acetonitrile) from 70-100% B in 20 min and help up to 25 min at the flow rate of 1 ml/min. Peak areas were calculated and the concentrations of ester were determined from the standard curve.

2.2.2 *In vitro* cell cytotoxicity and melanogenesis induction assays of the saturated fatty acid methyl esters

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2.2.2.1 Cell culture

B16F10 melanoma cells were cultured in 75-cm^2 flask in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a humidified incubator with 5% carbon dioxide (CO_2). Cells were grown to semiconfluence and harvested by 0.25%, w/v trypsin and 0.06 mM ethylenediamine tetraacetic acid (EDTA) in phosphate buffer saline (PBS). Cells were resuspended in the complete DMEM and the cell numbers were counted by using a hemacytometer. All experiments were performed in triplicate.

2.2.2.2 Sample preparation

The saturated fatty acid methyl esters and the corresponding saturated fatty acids at the concentrations range of 2.5-50.0 μg/ml were dissolved in absolute ethanol. Theophylline, a cAMP-elevating agent, at 2.5, 5.0 and 10.0 μg/ml was used as a positive control.

2.2.2.3 Cell cytotoxicity by SRB assay

The sulforhodamine B (SRB) assay was used for cell cytotoxicity determination[160]. Briefly, cells were seeded at the density of 1×10^4cells/well in 96-well plates and incubated for 24 h. Cells were then treated with different concentrations of saturated fatty acid methyl esters, saturated fatty acids, theophylline and absolute ethanol (control). After 72-h incubation, the adherent cells were fixed with 50% trichloroacetic acid, washed and then dyed with SRB. The bound dye was solubilized and the absorbance was measured at 540 nm using a microplate reader. The percentages of cell viability were calculated according to the following equation:

$$\%\ \text{Cell viability} = (A/B)\times 100$$

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Where A was the absorbance of the sample and B was the absorbance of the control (absolute ethanol).

2.2.2.4 Melanin content measurement

The melanin content measurement was performed according to the method previously described with slight modification[165]. Briefly, cells at the density of 10×10^4cells/well were plated in 6-well plates and incubated overnight for cell adhesion. Various concentrations of the saturated fatty acid methyl esters, saturated fatty acids, theophylline and absolute ethanol were then added and incubated for 72 h. After removing the medium and washing with PBS, the cells were dissolved in 500 μl of 2 N sodium hydroxide for 1 h at 60°C. The absorbance was measured at 450 nm using a microplate reader and the melanin content was determined in comparing to the standard synthetic melanin. The total protein content of the sample was measured by the Bradford dye-binding method using bovine serum albumin as a standard[173]. For the determination of the actual melanin formation from the same numbers of cells, the melanin content of each treatment was divided by the total protein content. The percentages of the relative ratio of melanin content was calculated as the following formula:

$$\% \text{ Relative ratio of the melanin content} = (Mt/Mc) \times 100$$

Where Mt was the melanin content of the sample divided by the total protein content of the sample and Mc was the melanin content of the control divided by the total protein content of the control (absolute ethanol).

2.2.2.5 Tyrosinase activity measurement

Tyrosinase activity was analyzed by the method described by Ohguchi et al. with slight modification[167]. Briefly, the treated cells were washed with ice-cold

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PBS, lysed with lysis reagent containing protease inhibitors and then incubated at 4°C for 30 min. The lysates were centrifuged at 12,000 rpm for 10 min. The obtained supernatants were collected and then mixed with the mixture containing 50 mM phosphate buffer (pH 6.8) and 0.05% *l*-dopa. The reaction mixtures were incubated for 2 h at 37°C. Then, the dopachrome formation was measured for the absorbance at 490 nm using a microplate reader. The enzyme activity was calculated in comparing to the standard mushroom tyrosinase. The total protein content of sample was also evaluated. Enzyme activity of the sample was compared to the control and calculated as the percentage of the relative ratio of the enzyme activity as the following equation:

$$\% \text{ Relative ratio of enzyme activity} = (Tt/Tc) \times 100$$

Where Tt was the tyrosinase activity of the sample divided by the total protein content of the sample and Tc was the tyrosinase activity of the control divided by the total protein content of the control (absolute ethanol).

2.2.2.6 Selection of the saturated fatty acid methyl ester to load in the selected blank niosomes

The saturated fatty acid methyl ester that showed the lowest cytotoxicity and the highest melanin induction activity was selected to load in the selected blank niosomes for the further study.

2.2.3 Development of the blank niosomes

2.2.3.1 Formulations of the blank niosomes

Table 6 showed the compositions of twelve blank niosomal formulations. These formulations were classified into 3 types, which were neutral, cationic and anionic niosomes, based on the addition and non-addition of the

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Table 6 Niosomal compositions of twelve blank niosomes prepared by the chloroform film method with sonication

Formula no.	Type of niosomes	Niosomal compositions	
		compositions	molar ratio
1	Non-ionic	Span 60:cholesterol	7:3
2	Non-ionic	Tween 61:cholesterol	1:1
3	Non-ionic	Tween 61:cholesterol	7:3
4	Non-ionic	Brij 72:cholesterol	7:3
5	Cationic	Span 60:cholesterol:DDAB	7:3:0.75
6	Cationic	Tween 61:cholesterol:DDAB	1:1:0.3
7	Cationic	Tween 61:cholesterol:DDAB	7:3:1.5
8	Cationic	Brij 72:cholesterol:DDAB	7:3:0.65
9	Anionic	Span 60:cholesterol:DP	7:3:0.75
10	Anionic	Tween 61:cholesterol:DP	1:1:0.3
11	Anionic	Tween 61:cholesterol:DP	7:3:1.5
12	Anionic	Brij 72:cholesterol:DP	7:3:0.65

charged molecule, either positively charged (dimethyl dioctadecyl ammonium bromide; DDAB) or negatively charged (dicetyl phosphate; DP) molecule. Twenty millimolar of each formulation was prepared by the chloroform film method with sonication. The niosomal compositions were mixed and dissolved in chloroform. The solvent was evaporated by a rotary evaporator at room temperature (30±2°C) to obtain a thin film. The residual solvent was dried overnight in a vacuum desiccator. The film was then suspended in distilled water and swirled in a water bath at 50±2°C for 15 min. The obtained dispersion was sonicated using a microtip probe sonicator for 6 min at the pulse on 3.0 and pulse off 1.0, 35% amplitude. The dispersion was then centrifuged at 7,000 rpm for 1 min to remove the traces of titanium from the sonication step.

2.2.3.2 Physical characteristics of the blank niosomes

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Physical characteristics and stability of the twelve blank niosomes were investigated when kept in the vials (5 ml of each blank niosomal dispersion) at 4±2, room temperature (30±2) and 45±2°C for 3 months. At 0, 1, 2 and 3 months, the physical appearances, including pH, color and sedimentation, were observed. The vesicular size and polydispersity index were measured at 25°C by a dynamic light scattering using the nonnegative constrained least squares algorithm mode particle size distribution analysis. The measurements were performed in triplicate with five cycles in each measurement. The measurement conditions were set at 30 s with 10 s pulse between the cycles. The zeta potential values of all samples were obtained by the phase analysis light scattering software. The time-dependent correlation function on the scattered light intensity was measured at a scattering angle of 90°. The measurements were performed in triplicate with twelve cycles in each measurement.

2.2.3.3 Selection of the blank niosomes to load with the selected saturated fatty acid methyl ester

Three different charged blank niosomal formulations that exhibited high physical stability in the nanosize range were selected to load with the selected saturated fatty acid methyl ester.

2.2.4 Development of the selected saturated fatty acid methyl ester loaded in the selected niosomes

2.2.4.1 Preparation of myristic acid methyl ester or methyl myristate loaded in the selected niosomes

Twenty millimolar of three niosomal formulations (neutral, cationic and anionic niosomes) loaded with myristic acid methyl ester or methyl myristate (MM)

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were prepared by the chloroform film method with sonication. The neutral niosomes were composed of Brij72 mixed with cholesterol at 7:3 molar ratio, while the cationic and anionic niosomes were consisted of Brij72/cholesterol/DDAB at 7:3:0.65 molar ratio and Brij72/ cholesterol/DP at 7:3:0.65 molar ratio, respectively. The niosomal compositions together with MM were mixed and dissolved in chloroform. Then, the mixture was processed to obtain the niosomal dispersion as previously described in 2.2.3.1.

2.2.4.2 Maximum loading of MM in the selected niosomes

MM was loaded in three niosomes with the concentrations increased from 0.1 to 20%, w/w in order to determine the maximum loading of MM in the niosomes. The maximum loading was the concentration of MM loaded in niosomes that gave no precipitation.

2.2.4.3 Entrapment efficiency of MM at the maximum loading concentrations in the selected niosomes

The loaded MM in each niosomal formulation at the maximum loading was separated from the free MM by gel filtration using Sephadex G-50 as a packing material and distilled water as an eluent. Eluants were collected in fractions using a fractional collector at the flow rate of 1 ml/min. The fraction was collected at 5 ml per tube. The fractions containing the MM loaded niosomes detected at 470 nm were pooled and dried with a lyophilizer[198]. The fractions containing the free MM detected at 214 nm were pooled and dried with a lyophilizer. The dried niosomal fractions were dissolved in methanol and analyzed for MM contents by HPLC as previously described in 2.2.1.4. Peak areas were calculated and the concentrations of MM were determined from the standard curve (y = 895636x, R^2 = 0.999). Entrapment

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efficiency of MM loaded in niosomes was calculated according to the following equation:

$$\% \text{ Entrapment efficiency} = \frac{\text{amount of MM in the niosomes}}{\text{the initial loading amount of MM}} \times 100$$

2.2.4.4 Morphological study of the selected niosomes loaded with MM

The morphology of the blank and MM loaded niosomes were investigated by transmission electron microscope (TEM) using negative staining technique. A drop of the dispersion was applied on a 300-mesh formvar copper grid on paraffin and allowed the sample to adhere for 10 min. The remaining dispersion was removed and a drop of aqueous solution of ammonium molybdate (2%, w/v) was applied for 5 min. The remaining solution was then removed, air dried and examined with a TEM.

2.2.4.5 Physical and chemical stability of MM loaded in the selected niosomes

MM loaded in various charged niosomes (5 ml) were kept in vials and stored at 4±2, room temperature (30±2) and 45±2°C for 3 months. At 0, 1, 2 and 3 months, the physical appearances (pH, color and sedimentation) of the samples were observed. The samples were withdrawn for vesicular size, polydispersity index, zeta potential and the MM contents. The size, polydispersity index and zeta potential of vesicles were measured at 25°C by a dynamic light scattering as previously described in 2.2.3.2. The MM contents in the niosomes were determined by HPLC as described in 2.2.1.4.

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2.2.5 *In vitro* cell cytotoxicity, melanogenesis induction activity and

transfollicular penetration assays of MM loaded in the selected niosomes

2.2.5.1 Cell culture

Fibroblasts derived from the skin specimen of a dental patient at 6th passage were obtained from Faculty of Dentistry, Chiang Mai University in Thailand. Both human skin fibroblasts and B16F10 melanoma cells were cultured in 75-cm^2 flask in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified incubator with 5% CO_2. Cells were counted by a hemacytometer. All experiments were performed in triplicate.

2.2.5.2 Sample preparation

The free MM at 0.33, 13.60 and 34.80 μM was dissolved in absolute ethanol. The blank and MM loaded in various charged niosomes were filtered through 0.2 μm filter membrane for sterilization. Theophylline at 0.28 mM was dissolved in the culture medium and filtered for sterilization.

2.2.5.3 Cell cytotoxicity by SRB assay in human skin fibroblasts and B16F10 melanoma cells

SRB assay was used to evaluate the cytotoxic effect in human skin fibroblasts and B16F10 melanoma cells[160]. The fibroblasts at the 6th passage and B16F10 melanoma cells were used at the same density of 1×10^4cells/well. Various concentrations of the free MM (0.33, 13.60 and 34.80 μM), the blank and MM loaded in niosomes at 0.20 mM, theophylline at 0.28 mM and the solvent (absolute ethanol or culture medium) were tested by the SRB assay with the same method as in 2.2.2.3. The cell viability as well as the cytotoxic ratio of cell viability in human skin fibroblasts and B16F10 melanoma cells were calculated. The cytotoxic ratio of cell viability in human skin fibroblasts and B16F10 melanoma cells was calculated as the

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following formula:

$$\text{Cytotoxic ratio} = \frac{\text{\% cell viability in human skin fibroblasts}}{\text{\% cell viability in B16F10 melanoma cells}}$$

2.2.5.4 Melanin content measurement

Various concentrations of the free MM (0.33, 13.60 and 34.80 μM), the blank and MM loaded in niosomes at 0.20 mM, theophylline at 0.28 mM and the solvent (absolute ethanol or culture medium) were investigated for the melanin stimulation activity in B16F10 melanoma cells with the same method as in 2.2.2.4.

2.2.5.5 Tyrosinase activity measurement

The tyrosinase activity of the free MM (0.33, 13.60 and 34.80 μM), the blank and MM loaded in niosomes at 0.20 mM, theophylline at 0.28 mM and the solvent (absolute ethanol or culture medium) were performed in B16F10 melanoma cells with the same method as in 2.2.2.5.

2.2.5.6 Tyrosinase-related protein-2 activity measurement

The assay of the TRP-2 activity was performed by the method described by Barber et al. with slight modification[169]. Briefly, the supernatant obtained from the lysis of the treated cells was mixed with the mixture consisting of 1 mM phenylthiourea, 2 mM EDTA and 10 mM sodium phosphate buffer (pH 6.8). Dopachrome solution, which was separately prepared by mixing an equal volume of 1 mM *l*-dopa and 2 mM sodium periodate, was added into the mixture and incubated at 37°C for 2 h. The decreased absorbance based on the consumption of the dopachrome was measured at 490 nm. The reaction mixture with bovine serum albumin instead of the cell supernatant was used as a negative control. The total protein content of the treated cells was also evaluated. The TRP-2 activity of the

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sample was compared with the control and calculated as the percentage of the relative ratio of the TRP-2 activity according to the following equation:

% Relative ratio of TRP-2 activity = $(Dt/Dc) \times 100$

Where Dt was the TRP-2 activity of the sample divided by the total protein content of the sample and Dc was the TRP-2 activity of the control divided by the total protein content of the control.

2.2.5.7 Transfollicular penetration

A. Skin sample

The porcine skin was freshly taken from the male newborn landrace pigs (1.0–1.5 kg) which died of natural causes few days after birth and obtained from Yupa farm at Doi Saket, Chiang Mai, Thailand during November - December, 2011. The *in vitro* transfollicular delivery protocol in porcine skin was reviewed and approved by the ethical committee of Faculty of Medicine, Chiang Mai University in Thailand (Protocol Number: 25/2554).

B. Preparation of the porcine skin

The newborn porcine back skin was shaved and carefully separated. The excess subcutaneous fat was then carefully removed using a scalpel. For cyanoacrylate skin surface stripping, a drop of the power glue (99.0%, w/w cyanoacrylate) was placed on the skin and covered with a glass slide under slight pressure as previously described[199]. After the polymerization of glue, the glass slide was removed with one quick movement to open all hair follicles.

C. Selective follicular blocking technique

The number of hair follicles on the porcine skin was counted. A pair of the skin sample with an equal number of hair follicles was used as opened and

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blocked hair follicle skin sample. All hair follicles of the blocked hair follicle skin were closed using the follicular closing technique with some modification[156]. Briefly, each follicular orifice of the blocked hair follicle skin was carefully closed with the small amount of the nail varnish using a blunt 30 gauge needle and dried for the complete closing of the follicular shunt. This technique was able to avoid the interference of the intercellular penetration areas.

D. Sample preparation

The samples used for the transfollicular penetration study were MM solution in 70% (v/v) propylene glycol and MM loaded in neutral, cationic and anionic niosomes. All formulations contained 2 mg/ml of MM.

E. Transfollicular penetration study

The opened and blocked hair follicle skin samples were mounted on the receiver compartment of the Franz diffusion cells with the viable epidermis side facing upwards to the donor compartment. The available diffusion area of the porcine skin was 2.46 cm^2. The receiver chamber was filled with 14 ml of phosphate buffer saline (pH 7.4), constantly stirred at 100 rpm with a small magnetic bar and the temperature was controlled at 37$\pm$2°C throughout the experiment. An amount of 1,000 μl of the samples was placed into the donor compartment and covered with a paraffin film. Cells were stopped at 1, 2, 4 and 6 h. The treated porcine skin samples were removed and swung twice in 100 ml of distilled water. The skin was stripped with an adhesive tape using 3 M Scotch® Magic™ tape (1 cm × 1 cm)[200]. Each tape was charged with a weight of 300 g for 10 s and then removed rapidly[201]. The stripping tapes were discarded to remove excess samples[202]. The MM content of each sample was extracted from the skin. The stripped skin was cut into small pieces,

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extracted twice with 1 ml of dichloromethane, sonicated for 10 min in an ice bath and filtered. The dichloromethane extract containing MM was evaporated to dryness under reduced pressure. Methanol was then added to dissolve MM in the extract and filtered through 0.45 μm filter membrane for HPLC determination. For the receiver compartment, the receiving solution was freeze-dried and reconstituted with 1 ml of methanol. The MM content was determined by HPLC as the previous method in 2.2.1.4. The experiments were done in triplicate.

F. Analysis of transfollicular penetration

The cumulative amounts, fluxes and transfollicular penetration per one hair follicle (HF) both in the skin and the receiving solution were calculated by the following equations:

$$\text{Cumulative amounts } (\mu g/cm^2) = \frac{(\text{MMopen - MMblock})}{2.46}$$

$$\text{Fluxes } (\mu g/cm^2/h) = \frac{(\text{MMopen - MMblock})}{2.46 \times 6}$$

Transfollicular penetration per one hair follicle (μg/one HF)

$$= \frac{(\text{MMopen} - \text{MMblock})}{\text{number of HF}}$$

Where MMopen was the MM contents in the opened system, MMblock was the MM contents in the blocked system and HF was the hair follicle.

2.2.5.8 Selection of MM loaded in the selected niosomes to incorporate in the hair lotion formulation

The MM loaded in niosomes that demonstrated the highest melanin induction activity, transfollicular penetration and good physicochemical properties

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were selected to incorporate in the hair lotion formulation.

2.2.6 Development of the hair lotion bases

2.2.6.1 Interaction study of the non-ionic surfactants and the MM loaded in the selected niosomes

The high HLB non-ionic surfactants were investigated their interaction with MM loaded in cationic niosomes to use as the wetting agent in the hair lotion formulation. The 0.1% of MM loaded in cationic niosomes and nine non-ionic surfactant solutions, including polyethylene glycol-60-hydrogenated castor oil (HC-60), polyethylene glycol-100-hydrogenated castor oil (HC-100), polyoxyethylene-20-sorbitan monostearate (TS-10V), polypropylene glycol-6-decyltetradeceth-20 (PEN-4620), polyethylene glycol-20-glyceryl triisostearate (GT-20IS), polyglyceryl-10-isostearate (Decaglyn 1-IS), polyglyceryl-10-diisostearate (Sunsoft Q-192Y), saccharose monolaurate (L-1695) and polyoxyethylene-20-sorbitan monolaurate (Tween20), were separately prepared. The diluted MM loaded in niosomal dispersion was then mixed with the non-ionic surfactant solution and incubated on the shaker at 37±2°C for 1 h. The turbidity and the vesicular size of the mixture were measured by the UV-vis spectrophotometer at 450 nm and the dynamic light scattering, respectively[203-204]. The percentage changes of turbidity and vesicular size of mixture were compared to the control.

2.2.6.2 Selection of the non-ionic surfactants for hair lotion base formulations

The non-ionic surfactants that changed the turbidity and vesicular size less than ±15.0% were chosen to incorporate in the hair lotion base formulations.

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2.2.6.3 Preparation of the hair lotion base formulations

Table 7 showed the compositions of the fifteen hair lotion base formulations. Each hair lotion base formulation contained different concentrations of the selected non-ionic surfactant system (Table 8). The MM loaded in cationic niosomal dispersion was added in place of water in the base formulations. Briefly, the hair lotion base compositions were weighed and separately mixed in each part. In part C, carboxy methyl cellulose (CMC) and hydroxy propyl methyl cellulose (HPMC) were dispersed in propylene glycol on the water bath at 70±2°C. After the premixing, part B was added into part A and homogeneously mixed. Part C was cooled down at the room temperature (30±2°C) and then added into the mixture. The hair lotion base formulations were stored at room temperature (30±2°C) overnight and the physical characteristics and thermodynamic stability were investigated.

2.2.6.4 Physical characteristics and thermodynamic stability of the hair lotion base formulations

The developed hair lotion base formulations were evaluated for the physical characteristics, including appearance, odor, pH, specific gravity, viscosity, vesicular size, polydispersity index and zeta potential. Then, ten milliliters of each hair lotion base were kept in a bottle and investigated for thermodynamic stability by the heating and cooling method at 45 and 4°C for 6 cycles. The base formulations that showed thermodynamic stable at these temperatures were investigated for physical characteristics. Only stable base formulations were compared to the initial data and selected for the further study.

2.2.6.5 Selection of the hair lotion base formulation to incorporate the MM loaded and not loaded in cationic niosomes

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Table 7 Compositions of the hair lotion base formulations containing different concentrations of the non-ionic surfactant system

Ingredients \ Formula No.	1 (%w/w)	2 (%w/w)	3 (%w/w)	4 (%w/w)	5 (%w/w)	6 (%w/w)	7 (%w/w)	8 (%w/w)	9 (%w/w)	10 (%w/w)	11 (%w/w)	12 (%w/w)	13 (%w/w)	14 (%w/w)	15 (%w/w)
Phase A															
Jojoba oil	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Non-ionic surfactant (s)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	1.25	1.25	1.25	1.25	5.0	2.5
Vitamin E	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Absolute ethanol	10.0	10.0	10.0	10.0	10.0	10.0	-	-	-	10.0	10.0	-	5.0	-	2.0
Glycerin	-	-	-	-	-	-	10.0	10.0	10.0	-	-	10.0	-	10.0	8.0
Phase B															
Trimethyl glycine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Panthenol	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Liquid germall plus®	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Cationic niosomes loaded with MM	q.s to 100.0	q.s to 100.0	q.s to 100.0	q.s to 100.0	q.s to 100.0	q.s to 100.0	q.s to 100.0	q.s to 100.0	q.s to 100.0	q.s to 100.0	q.s to 100.0	q.s to 100.0	q.s to 100.0	q.s to 100.0	q.s to 100.0
Phase C															
CMC	0.8	0.8	-	0.6	0.6	-	0.7	0.7	-	0.6	0.6	-	1.2	0.2	0.4
HPMC	-	0.8	0.3	-	0.2	0.8	-	0.2	1.0	-	0.3	0.8	-	-	-
Propypene glycol	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	6.0	6.0	6.0	5.0	5.0	5.0
	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

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Table 8 Concentrations of the non-ionic surfactant system used in the hair lotion base formulations

Hair lotion base Formula No.	Percentages of the non-ionic surfactant system (%)		Total percentages of the non-ionic surfactant system (%)
	A	B	
1	HC-60 1.25	PEN-4620 1.25	2.5
2	HC-60 1.25	PEN-4620 1.25	2.5
3	HC-60 1.25	PEN-4620 1.25	2.5
4	-	PEN-4620 2.5	2.5
5	-	PEN-4620 2.5	2.5
6	-	PEN-4620 2.5	2.5
7	HC-100 1.25	PEN-4620 1.25	2.5
8	HC-100 1.25	PEN-4620 1.25	2.5
9	HC-100 1.25	PEN-4620 1.25	2.5
10	HC-60 1.25	-	1.25
11	HC-60 1.25	-	1.25
12	HC-60 1.25	-	1.25
13	Tween20 1.25	-	1.25
14	HC-100 5.0	-	5.0
15	HC-100 2.5	-	2.5

The hair lotion base formulation that gave good physical stability was selected to incorporate with the MM loaded and not loaded in cationic niosomes for the further study.

2.2.7 Development of the hair lotion containing MM loaded and not loaded in cationic niosomes

2.2.7.1 Preparation of the hair lotion containing MM loaded and not loaded in cationic niosomes

Table 9 showed the compositions of the selected hair lotion base. MM not loaded in cationic niosomes was added in place of cationic niosomes loaded with

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Table 9 Compositions of the selected hair lotion formulation containing MM loaded and not loaded in cationic niosomes and the base formulation

Ingredients	Hair lotion base (%w/w)	Hair lotion containing MM (0.7 mg/ml) (% w/w)	Hair lotion containing MM loaded in cationic niosomes (0.7 mg/ml) (%w/w)
Phase A			
Jojoba oil	0.1	0.1	0.1
HC-60	1.25	1.25	1.25
Vitamin E	0.1	0.1	0.1
Glycerin	10.0	10.0	10.0
MM	-	0.07	-
Phase B			
Trimethyl glycine	0.5	0.5	0.5
Panthenol	0.5	0.5	0.5
Liquid germall plus®	0.4	0.4	0.4
MM loaded in cationic niosomes (0.7 mg/ml)	-	-	q.s. to 100.0
Distilled water	q.s. to 100.0	q.s. to 100.0	-
Phase C			
HPMC	0.8	0.8	0.8
Propylene glycol	6.0	6.0	6.0
	100.0	100.0	100.0

MM in the formulation (0.7 mg/ml). The final concentration of MM in both hair lotions containing MM loaded and not loaded in cationic niosomes was 0.7 mg/ml. The hair lotion base formulation was also prepared and the distilled water was added instead of the MM loaded and not loaded in niosomes.

2.2.7.2 Morphological study of cationic niosomes loaded with MM in the hair lotion

The morphology of the cationic niosomes loaded with MM in the hair lotion was investigated by TEM as the method previously described in 2.2.4.4.

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2.2.7.3 Physical and chemical stability of MM loaded and not loaded in cationic niosomes incorporated in the hair lotion

An aliquot of the hair lotions composing of free MM and that loaded in cationic niosomes (5 ml) were kept in vials and stored at 4±2, room temperature (30±2) and 45±2°C for 3 months. At 0, 1, 2 and 3 months, the physical appearances (pH, color sedimentation and phase separation) of the samples were observed and the samples were withdrawn for vesicular size, zeta potential, polydispersity index and the MM contents with the same methods as in 2.2.3.2 and 2.2.1.4, respectively.

2.2.8 *In vitro* transfollicular penetration and *in vivo* rabbit skin irritation and melanogenesis induction of the hair lotion containing MM loaded and not loaded in cationic niosomes

2.2.8.1 *In vitro* transfollicular penetration of the hair lotion containing MM loaded and not loaded in cationic niosomes

A. Skin sample

The porcine skin was obtained from the male newborn landrace pigs from Yupa farm at Doi Saket, Chiang Mai, Thailand in May, 2012. The skin samples were prepared by cyanoacrylate skin surface stripping and the hair follicles were selectively blocked by the application of nail varnish as previously described in 2.2.5.7.

B. Sample preparation

The hair lotions containing MM loaded and not loaded in cationic niosomes were evaluated for transfollicular penetration study. All formulations contained 2 mg/ml of MM.

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C. Transfollicular penetration study

The transfollicular penetration of the hair lotion containing MM loaded and not loaded in cationic niosomes were performed with the same method as described in 2.2.5.7.

2.2.8.2 *In vivo* rabbit skin irritation by the single closed patch test

Three male rabbits (New Zealand White, 1.5–2.5 kg) purchased from the Department of Animal and Aquatic Science, Faculty of Agriculture, Chiang Mai University in Chiang Mai, Thailand were carefully kept for an acclimation period of 7 days in the limited-access rodent facility with the environmental conditions set at 25 ± 2°C, 60–90% RH and 12 h light/12 h dark cycle. The animals accessed to the rabbit-diet *ad libitum* and the drinking water was supplied to each cage. The dorsal area of the trunk of the rabbits was shaved to be free of fur with an electric clipper 24 h before the sample application. The shaved area of each animal was divided into 12 test sites with the area of 2.5 × 2.5 cm. An amount of 500 μl of the sample, including hair lotion containing MM loaded or not loaded in cationic niosomes, hair lotion base, blank cationic and MM loaded in cationic niosomes, MM at 1 mg/ml in absolute ethanol, absolute ethanol and 20% w/w sodium lauryl sulfate, was applied on each test area. The untreated area was used as a negative control. The treated areas were covered with the gauze patch and held with the non-irritating tape. After 4 h, the gauze patch and the test samples were removed and the treated areas were then washed with distilled water for 2 times and air dried. All test areas of the animals were visually examined and measured by Mexameter® for signs of erythema and edema at 1, 24, 48 and 72 h after patch removal. The skin reactions were graded and recorded according to the grades described in the OECD Guideline method 404[176].

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The Primary Irritation Index (PII) was calculated according to the following formula:

$$PII = \frac{[(\Sigma \text{ erythema grades at } 1,24,48,72 \text{ h} + \Sigma \text{ edema grades at } 1,24,48,72 \text{ h})]}{(\text{number of animals} \times 4 \text{ scoring intervals})}$$

The irritation degree was categorized based on the PII values as non-irritation (PII = 0), slight (PII = 0.01–1.99), moderate (PII=2.00–5.00) or severe (PII=5.01–8.00) irritation[177]. The study protocol was reviewed and approved by the ethical committee of Faculty of Medicine, Chiang Mai University in Thailand (Protocol Number: 25/2554).

2.2.8.3 *In vivo* melanogenesis induction in aged mice

A. Animals

The 4-week-old C57BL/6 black male mice purchased from the National Laboratory Animal Center, Mahidol University in Nakhon Pathom, Thailand were housed individually in the stainless steel cages in a room with controlled temperature (25±2°C), a 12 h cycle of light and dark and maintained on a standard laboratory diet and water *ad libitum* prior to the experiment. The mice were fed until the period of the appearance of gray body coat hairs which were about 9 months old.

B. Melanogenesis induction evaluation

Mice with age-induced gray body coat hairs, which indicated the initiation of the prolonged telogenic phase of the hair growth, were employed to assess the pigment-inducing activity of the samples. In this condition, the pigment recovery in the skin together with the fresh hair growth was considered to be the synonymous with the induced growth and melanogenesis of melanocytes. Any compound that could return the telogenic phase of hair growth to the anagenic phase (visualized by the change in the skin color with the growth of the black hair) would

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have the potential for hair repigmentation[38]. The dorsal of the aged C57BL/6 mouse skin was clipped to remove the gray body coat hairs. Four hair lotion formulations, including hair lotion base and those containing MM, MM loaded in cationic niosomes and theophylline (0.5 mg/ml), were applied (0.2 ml/cm^2) with gentle rubbing by using the finger tips twice a day for 2 months without intermission (3 animals/sample). The changes in the skin color and association of hair growth were monitored at the first day of each week and scored with the visual analog scale as the following: 0 = no change of the skin color, 1 = first sign of the skin color change with the bluish spot, 2 = the change of the skin color with bluish skin or hair growth with the white hairs of more than 25%, 3 = the change of the skin color or hair growth with the white hairs of 5-24% and 4 = the change of the skin color or hair growth with the white hairs of less than 5%[40]. The *in vivo* melanogenesis induction study protocol was reviewed and approved by the ethical committee of Faculty of Medicine, Chiang Mai University in Thailand (Protocol Number: 25/2554).

C. Histological examination

After sacrifice, the skin specimens obtained from the mice in each treated group were dissected out (4-6 mm and full depth including the subcutaneous tissue). The standard procedures were processed for histological examination and the paraffin sections with 5 μm thickness were prepared. The sections were processed for the melanin bleach staining. The histological examination was investigated using the light microscope equipped with the camera.

2.2.9 Statistical analysis

Data were expressed as the mean ± S.E. of three independent experiments. Statistical significant difference at *p*-value <0.05 was analyzed by using the one-way

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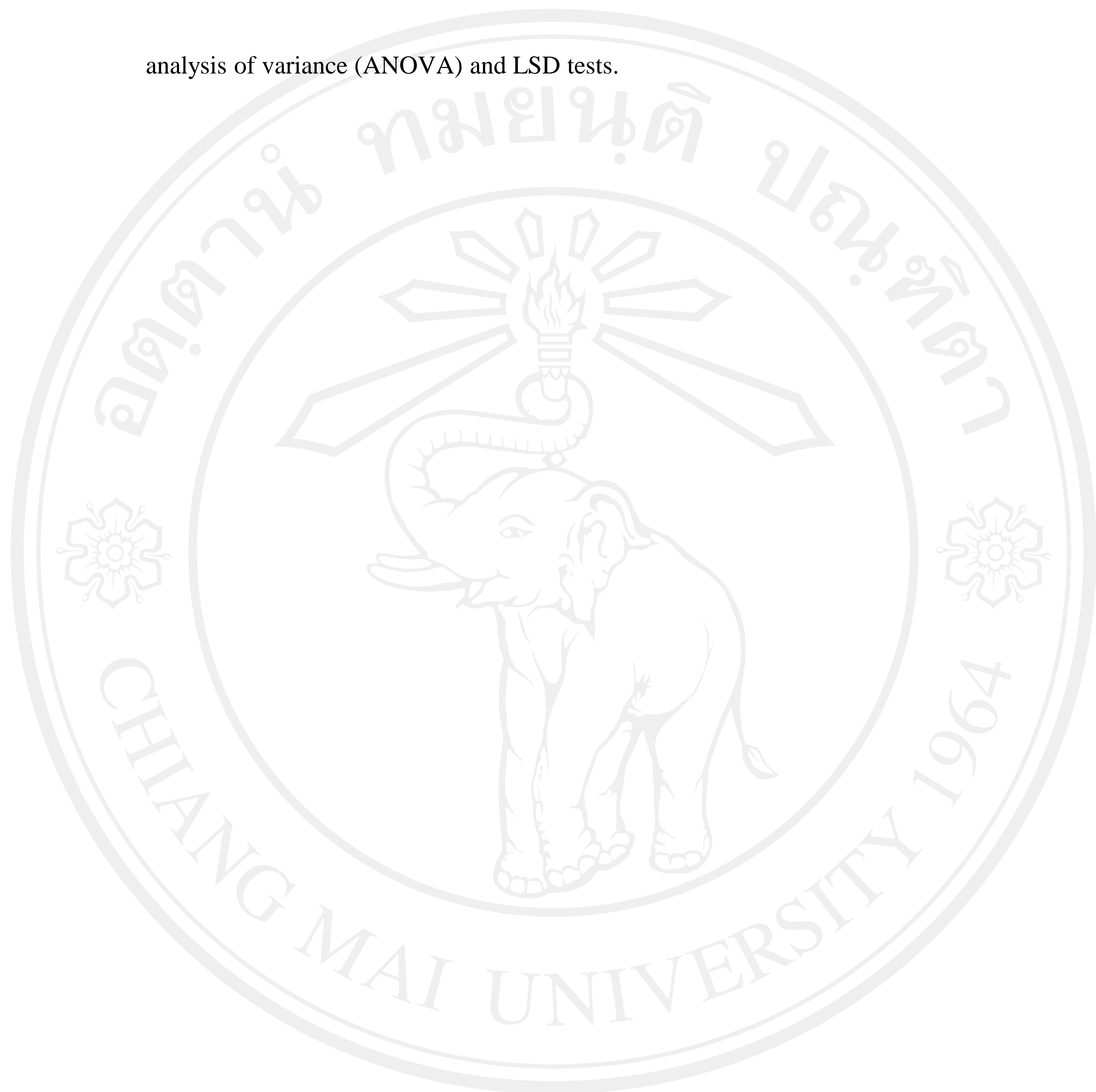
analysis of variance (ANOVA) and LSD tests.



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